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LOX-1 Is a Novel Therapeutic Target in Neonatal Hypoxic-Ischemic Encephalopathy

Tomohisa Akamatsu,*† Hongmei Dai,* Masashi Mizuguchi,‡ Yu-ichi Goto,* Akira Oka,† and Masayuki Itoh*

From the Department of Mental Retardation and Birth Defect Research, National Center of Neurology and Psychiatry, Kodaira, Tokyo; and the Departments of Pediatrics† and Developmental Medical Sciences,‡ Graduate School of Medicine, The University of Tokyo, Tokyo, Japan*

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Address correspondence to Masayuki Itoh, M.D., Ph.D., Department of Mental Retardation and Birth Defect Research, National Center of Neurology and Psychiatry, 4-1-1 Ogawa-higashi, Kodaira, Tokyo 187-8502, Japan. E-mail: itoh@ncnp.go.jp.

Neonatal hypoxic-ischemic encephalopathy (HIE) remains a serious burden in neonatal care. Hypothermia provides a good outcome in some babies with HIE. Here, we investigated the biological mechanisms of its neuroprotective effect and sought for a new therapeutic target. We made neonatal HIE rats and subjected some of them to hypothermia at 28°C for 3 hours. We pathologically confirmed the efficacy of hypothermia against the neonatal HIE brain. To clarify the molecular mechanism of hypothermia's efficacy, we analyzed mRNA expression, immunoassay, and pathology in the brain with or without HIE and/or hypothermia. We selected from these analyses 12 molecules with possible neuroprotective effects. After identification of lectin-like oxidized low-density lipoprotein receptor-1 (LOX-1) as a therapeutic target candidate, we examined the efficacy of an anti—LOX-1 neutralizing antibody in neonatal HIE rats. Administration of an anti—LOX-1 neutralizing antibody reduced infarction area, brain edema, and apoptotic cell death to a degree comparable with hypothermia. Protection from those pathological conditions was considered part of the therapeutic mechanism of hypothermia. The efficacy of administering anti—LOX-1 neutralizing antibody was similar to that of hypothermia. LOX-1 is a promising therapeutic target in neonatal HIE, and the inhibition of LOX-1 may become a novel treatment for babies who have experienced asphyxia. *(Am J Pathol 2014, 184: 1843—1852; http://dx.doi.org/10.1016/j.ajpath.2014.02.022)*

Neonatal hypoxic-ischemic encephalopathy (HIE) is a main cause of perinatal brain damage. The mortality ranges from 10% to 60%, and 25% of survivors have neurological sequel, such as cerebral palsy, mental retardation, deafness, and epilepsy.[1,2] Prevention and treatment of neonatal HIE remains to be established until now. Neonatal HIE occurs after asphyxia and is induced by the combination of intrinsic and environmental factors.[3,4] Although many animal experiments and clinical trials have been performed, much remains unsolved as to the pathophysiology, biology, and therapy of neonatal HIE.

Despite the remarkable progress in neonatal care for the past two decades, neonatal HIE has not shown a significant decline with an incidence of 1 to 6 per 1000 live births.[5] Novel pharmacological treatments, such as erythropoietin and xenon, have been attempted with babies who have experienced asphyxia, but they have not led to a substantial improvement.[6] Since the 1990s, hypothermia (HT) has been applied to babies who have experienced asphyxia as a new therapy. Three large-scale randomized controlled studies of HT for term infants with HIE reported that head or whole-body cooling significantly reduces neurological sequel.[7—9] HT exerts a neuroprotective effect by reducing energy consumption and by repressing glutamate release and free radicals.[10] Experimental HT for neonatal HIE involves many cytoplasmic cascades or pathways such as the excito-oxidative cascade that involves lactic acid, glutamate, nitric oxide, mitochondrial failure, free radicals, lipid peroxidation, and inflammation.[11—14] We previously demonstrated that

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edaravon, an antioxidative agent, is effective in a rat model of neonatal HIE with a significant reduction in neuronal apoptosis and infarct areas.[15] These biological pathways obviously conclude in apoptotic cell death.

By contrast, HT after brain injury is useful for controlling intracranial pressure caused by brain edema.[16] However, in the rewarming period, a rebound rise is often seen in intracranial pressure because of leakage from the blood-brain barrier (BBB), resulting in brain damage.[16] In the developing brain, the BBB is vulnerable to various stresses.[17] Protection against a BBB break is needed during the care of an immature brain. Taken together, it is important to rescue the asphyxiated baby brain by inhibiting the progress of brain edema and the activation of apoptotic cell death. Much remains to be elucidated as to the biological and molecular mechanisms of the neuroprotective effect of HT.

In the present study, we explored the molecular mechanism of the neuroprotective effect of HT against neonatal HIE and identified lectin-like oxidized low-density lipoprotein receptor-1 (LOX-1) as a new therapeutic target. Furthermore, we evaluated the effect of a new treatment by blocking LOX-1 in the asphyxiated neonatal brain.

Materials and Methods

All experiments were conducted according to the protocols approved by the Animal Experiment Ethical Committee of the National Center of Neurology and Psychiatry. Animals were housed under a 12-hour:12-hour light/dark cycle with food and water available *ad libitum*.

Animal Preparation and Neonatal HIE Model

We used Sprague-Dawley rat pups (CLEA Japan Inc., Tokyo, Japan) on postnatal day 7 in all experiments. In the initial experiments, pups were assigned to three groups: control (CTL), neonatal HIE, and HT. In addition, an experiment was conducted on other groups of pups for a therapeutic trial. The sample distribution is summarized in Supplemental Table S1. CTL pups were kept at 36°C for 3 hours and underwent neither surgical procedure nor hypoxic exposure. HIE pups received left common carotid artery ligation and were kept under 8% O_2 at 36°C for 2 hours [hypoxic-ischemic (HI) insult], according to Rice's method,[18] and then at 36°C for the next 3 hours, resulting in lesion in the left hemisphere. At first, to decide a suitable temperature for HT treatment, we experimented with HT pups at 28°C or 32°C after HI insult and performed mRNA expression assays (Supplemental Table S2). We confirmed that the number of 28°C HT-induced genes was similar to that of 32°C HT-induced genes (Supplemental Table S3). Moreover, the brain infarction area of 28°C HT pups was smaller than that of 32°C HT pups (data not shown). The 28°C HT experiment was adapted as HT in the study. HT pups were kept on HT of 28°C for 3 hours immediately after HI insult. Therapeutic trial pups were administered an agent immediately after HI insult. In each experiment, a temperature-monitored pup was kept at 36°C or 28°C by a controller (ACT-101B; Unique Medical, Tokyo, Japan).[19,20] After these procedures, all pups were kept with their mothers as usual (12-hour:12-hour light/dark cycle) until sacrifice.

Tissue Preparation and Pathological Analyses

Pup brains were removed at 3, 6, 24, 48, or 72 hours after the procedures of control, HI insult, HT, and therapeutic trial. After being anesthetized, they were perfused intracardially with PBS or 4% paraformaldehyde in PBS. For expression analyses, perfusion was done with PBS, and then the brains were stored at −80°C until use.

For histopathological analyses, the brains were perfused with paraformaldehyde, removed, additionally infiltrated with paraformaldehyde at 4°C for 24 hours, embedded in paraffin, and cut into coronal serial sections (4 μm thick). The sections were then subjected to various staining, such as Nissl staining, TUNEL assay, and *in situ* hybridization. To evaluate apoptotic cell death, TUNEL assay was performed with ApopTag Fluorescein *In Situ* Apoptosis Detection Kit (Millipore, Billerica, MA) and antifade solution that contained DAPI. We counted all $TUNEL^+$ cells and/or $DAPI^+$ cells in four 1-mm^2 fields in the left cortex at ×400 magnification. We calculated the ratio (apoptotic ratio) of the TUNEL and DAPI $double^+$ cell number to the $DAPI^+$ cell number and calculated the mean and SD from four fields in each animal. In addition, TUNEL sections were double-stained with anti-NeuN mouse antibody (Millipore) to identify neurons.

Moreover, we performed immunohistochemistry to confirm LOX-1 expression in brains. Coronal sections of frozen rat brains (10 μm thick) were stained with a chicken polyclonal LOX-1 antibody (provided by Dr. T. Sawamura) and a secondary antibody of Alex Fluor 488-conjugated donkey anti-chicken IgY (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA).[21] All fluorescence sections were observed with a fluorescent microscope (BX51; Olympus, Tokyo, Japan).

Evaluation of Brain Edema and Infarction

For brain edema evaluation, we measured the brain-water content. Fresh unfixed brains 24, 48, and 72 hours after HI insult were divided into the left and right hemispheres. Each hemisphere was weighed immediately after removal (wet weight), and, after drying in an oven at 85°C for 72 hours,the same hemisphere was weighed again (dry weight). The ratio of $[(\text{wet weight}) - (\text{dry weight})/(\text{wet weight})]$ was calculated as the water content ratio. The ratio of the lesional side water content ratio to the nonlesional side water content ratio was defined as an edema ratio and was used to compare the groups.

We evaluated the infarct size at the hippocampus and hypothalamus levels with Nissl staining. Nissl-stained sections were photographed at ×20 magnification with a microscope (BX51; Olympus), and ImageJ software, version

1.45 (NIH, Bethesda, MD) was used to measure the intact and whole area in both the lesional and nonlesional sides. Then we calculated the ratio of the lesional side intact area to the nonlesional side intact area as the ratio of the intact area.

Exploration of Molecules Accounting for the Effects of HT

We made protein extracts and mRNA extracts from the stored samples. For whole mRNA expression analysis, we performed microarray analysis of CTL, HIE, and HT brains, using GeneChip Rat Gene 1.0 ST Array (Affymetrix, Santa Clara, CA) according to the manufacturer's instructions. In the comparison of mRNA expression data between CTL and HIE brains, we selected genes of HIE/CTL > 3 as up-regulation, and HIE/CTL < 0.5 as down-regulation. To confirm the expression levels of the genes found in this study, we performed real-time quantitative PCR (qPCR) and *in situ* hybridization of more than four brains from each group and age, using TaqMan gene expression assays and master mix (Applied Biosystems, Carlsbad, CA) with Lightcycler 480 II (Roche, Basel, Switzerland). A standard reference gene of glyceraldehyde-3−phosphate dehydrogenase (*GAPDH*) was used as a normalization reference. Furthermore, we performed *in situ* hybridization analysis, using PCR DIG Probe Synthesis Kit (Roche). The probes' primer sequences were as follows: the forward primer of *P2ry13* (purinergic receptor P2Y, G-protein coupled 13), 5′-CCTGGGGCTGCTGTGGCATC-3′; the reverse primer of *P2ry13*, 5′-TTCGCCTCCAGCCGCTTGTG-3′; the forward primer of *Olr1* (oxidized low-density lipoprotein receptor 1), 5′-TGACCCTGCCATGCCATGCT-3′; and the reverse primer of *Olr1*, 5′-TGGGGATGGTGGAGGCCCTG-3′. Coronal sections at the level of the hippocampus and hypothalamus were hybridized at 55°C for 16 hours with the probes. The reacted chromogen on the sections was colored with alkaline phosphate-labeled anti-DIG antibody (Roche) and nitroblue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate.

We performed immunoblotting and ELISA analyses to evaluate protein expressions of CTL, HIE, HT, and treated brains. After protein extraction, 20 μg of protein in each lane was subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis, electroblotted onto a polyvinylidene fluoride membrane, and incubated with the primary antibodies on protein-transferred membranes overnight at 4°C. Proteins were detected with the secondary anti-rabbit IgG antibody and ECL Prime Western Blotting Detection Reagent (GE Healthcare, Piscataway, NJ). After detection of a specific band, the expression level was measured by a luminescent image analyzer (ImageQuant

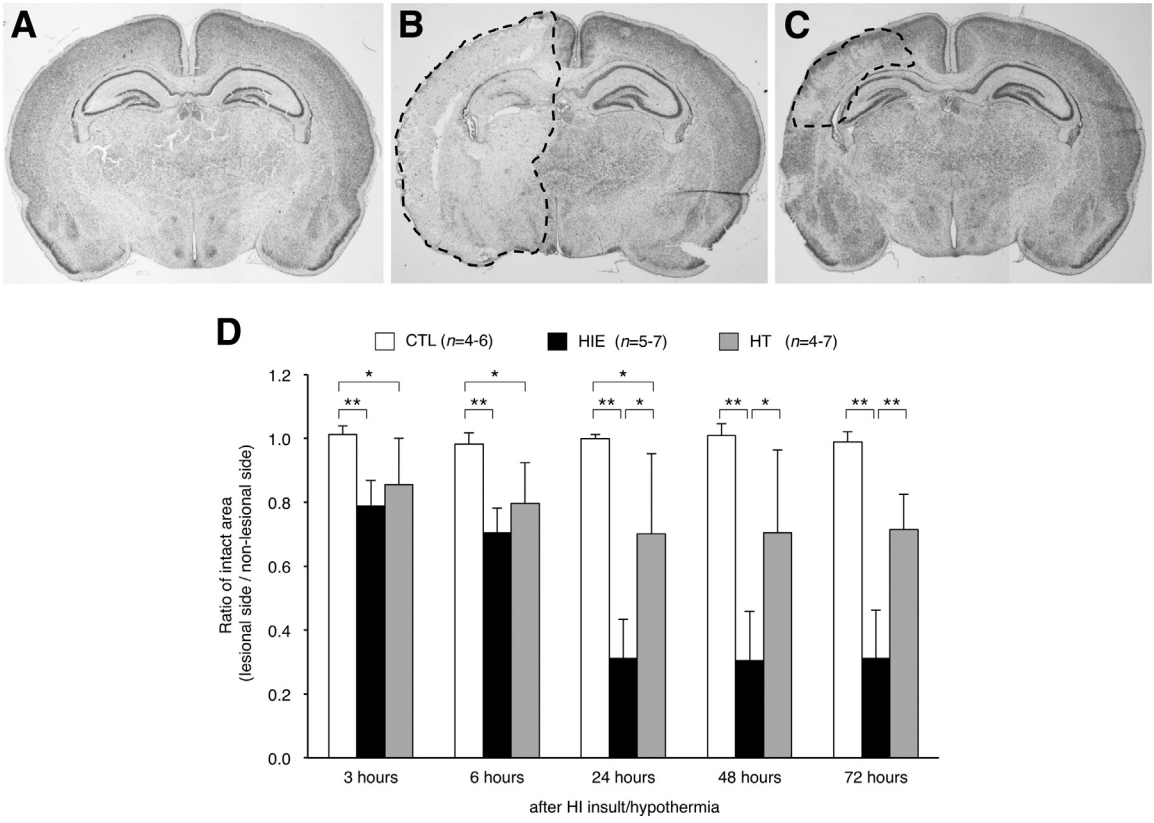

Figure 1 Differences among brains of the CTL (**A**), HIE (48 hours after HI insult; **B**), and HT (48 hours after HI insult; **C**) group. Each **dashed area** of the coronal sections with Nissl staining denotes infarction. **D:** The ratios of intact area are significantly lower in HIE than in CTL (3 to 72 hours after insult) and is significantly higher in HT than in HIE (24 to 72 hours). Data are expressed as means ± SD. $*P < 0.05$, $**P < 0.001$.

LAS 4000 mini; GE Healthcare) and normalized by GAPDH expression level. The primary antibodies were rabbit antibodies against cleaved caspase 3 (cCASP3; Sigma-Aldrich Co., St. Louis, MO), occludin (Zymed Laboratories Inc., Carlsbad, CA), ZO-1 (Zymed Laboratories Inc.), and GAPDH (Abcam, Cambridge, UK) as a normalization reference protein. We performed ELISA to measure malondialdehyde (MDA), by using the Rat MDA ELISA Kit (CUSABIO, Hubei, China). Samples were applied on precoated wells with the antibody for MDA. After the secondary reaction with the biotin-conjugated antibody, color intensities were measured with a spectrometer. Each sample was measured twice, corrected by the protein concentration, and calculated to obtain the mean. The mean was defined as the MDA level.

Antibody Treatment Targeting a New Therapeutic Candidate

As a therapeutic candidate, we selected LOX-1. An anti–LOX-1 neutralizing antibody (R&D Systems, Minneapolis, MN), 6 μg/kg or 60 μg/kg in PBS, was intraperitoneally administered immediately after HI insult. We also made the PBS-treated group, which was injected with 0.1 mL of the vehicle, PBS. Anti–LOX-1 neutralizing antibody or PBS was injected twice a day until sacrifice.

Statistical Analysis

All data were expressed as means ± SD. Data among multiple groups were analyzed with analysis of variance,

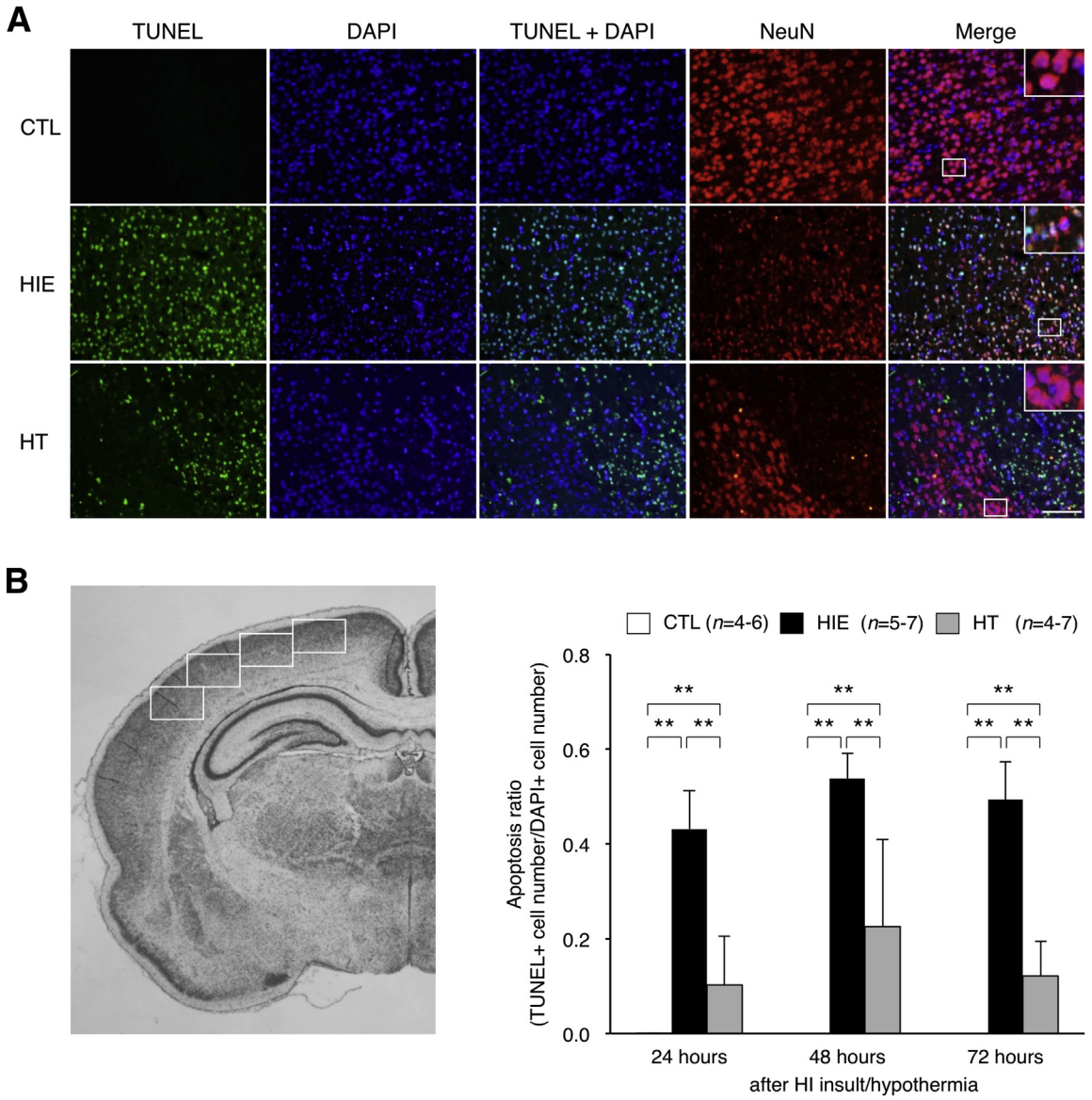

Figure 2 Reduction of apoptosis due to HT. **A:** HIE cortex shows numerous TUNEL$^+$ cells, most of which are NeuN$^+$. **Insets** are high-power views of rectangular areas. **B:** In four areas of each cortex (**rectangles**), the apoptosis ratio was calculated. The ratio was 0.4 to 0.6 in HIE brain and was reduced to 0.1 to 0.2 in HT brain. Data are expressed as means ± SD. *$P < 0.05$, **$P < 0.001$. Scale bar = 100 μm.

and data of all pairs of groups were analyzed with *t*-test or *U*-test with Statcel 2 (OMS Publication, Saitama, Japan). $P < 0.05$ was considered to be statistically significant.

Results

Brain Damage of HIE and HT in Neonatal Rats

The sample distribution (Supplemental Table S1) indicated no significant difference between groups in terms of sex and weight (data not shown). Neonatal HIE models showed infarction in the lesional (left) hemisphere but not in the nonlesional (right) hemisphere (Figure 1). The lesional (left) side pathology, showing infarction and edema, became larger with time after HI insult, as found in our previous report.[15]

Changes in infarction size (ratio of intact area) and apoptotic neuron (apoptosis ratio) in the CTL, HIE, and HT groups 3, 6, 24, 48, or 72 hours after HI insult are shown in Figures 1 and 2. CTL brains showed no damage to the cerebral hemispheres throughout the period, whereas HIE brains showed a significant reduction in the ratio of intact area, which progressed rapidly from 3 to 24 hours after HI insult (Figure 1D). In HT brains, however, the extent of reduction was significantly lower than in HIE brains (Figure 1D).

Next, we compared the number of positive cells undergoing apoptosis in the three groups. No TUNEL$^+$ cells were found in CTL brains (Figure 2). In HIE brains, many TUNEL$^+$ cells were also positive for NeuN (Figure 2A). Apoptosis ratios at 24, 48, and 72 hours were 0.43 ± 0.08, 0.54 ± 0.05, and 0.49 ± 0.08, respectively. In HT brains, apoptosis ratios at 24, 48, and 72 hours were 0.10 ± 0.10, 0.23 ± 0.18, and 0.12 ± 0.07, respectively, and were significantly lower than those of HIE (Figure 2B). These results indicate that HT significantly decreased infarct areas and dramatically reduced the number of apoptotic cells after HI insult.

Identification of Therapeutic Candidate Molecule from HT

We searched for the molecule most effective for attenuating HI-induced brain damage by HT. To observe early changes in gene expression preceding the pathological changes, we checked microarray data of CTL, HIE, and HT brains 3 hours after HI insult (Supplemental Tables S2 and S3) and found seven down-regulated genes in neonatal HIE (HIE/CTL < 0.5); three of them were miRNA (Supplemental Table S2). Three of the seven genes were overexpressed during HT after HI insult; gene function was known for one gene only. We also found 28 genes up-regulated by HI insult (HIE/CTL > 3) (Supplemental Table S2). Twelve of them were less expressed in HT brains; 11 of them had known functions. As a result of qPCR of the 12 genes (Supplemental Figure S1), the expression level of *P2ry13* was significantly down-regulated 3 and 6 hours after HI insult and was up-regulated at 3 and 6 hours after HI insult with HT. The expression level of *Olr1* was significantly up-regulated 3 hours after HI insult and was down-regulated 3 hours after HI insult with HT. Expression levels of the other genes were not significantly different between the groups. The results of *in situ* hybridization for *P2ry13* and *Olr1* are shown in Supplemental Figure S2. *P2ry13*$^+$ and *Olr1*$^+$ cells

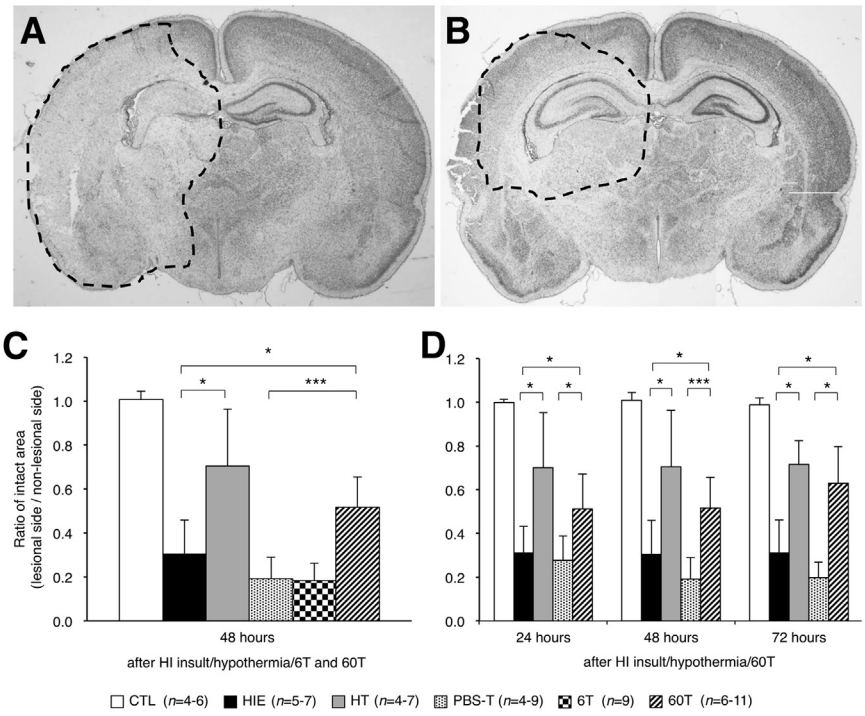

Figure 3 Pathological evaluation of anti—LOX-1 neutralizing antibody. The brain sections treated with anti—LOX-1 show marked reduction of infarction area (dashed areas) 48 hours after HI insult, compared with operated control (**A**, PBS-T brain; **B**, 60T brains). **C:** The ratio of intact area of 60T is significantly higher than in HIE, but that of 6T brains is not different from those of HIE and PBS-T brains (48 hours after HI insult). **D:** The efficacy of 60 μg/kg anti—LOX-1 treatment was noted from 24 hours after HI insult and was comparable with that of HT. Data are expressed as means ± SD. $*P < 0.05$, $***P < 0.001$.

were localized in the cerebral cortex, amygdala, hippocampus, thalamus, and hypothalamus. Many of the *P2ry13*$^{+}$ and *Olr1*$^{+}$ cells were neurons. Moreover, LOX-1was expressed in pyramidal neurons of the left lesional cortex (Supplemental Figure S3).

Effect of Anti—LOX-1 Neutralizing Antibody as a Therapeutic Candidate

From the expression analyses, we suspected that *P2ry13* was a neuroprotective factor and *Olr1* was a neurodamage-inducing factor. P2y13 is a purinergic receptor that is expressed in neurons and exerts an antioxidative effect.[22,23] LOX-1 (or Olr1) is a marker of myocardial infarction and vascular diseases.[24—27] We hypothesized that a neutralizing antibody to LOX-1, an antagonist of LOX-1, may have a therapeutic effect for neonatal HIE, because LOX-1 is expressed in neurons and is involved in vascular diseases.[27,28]

Before using the antibody, we confirmed that it binds to recombinant rat LOX-1 protein (Sino Biological Inc., Beijing, China) by immunoblotting (data not shown).[29] We administered 6 μg/kg or 60 μg/kg of anti—LOX-1 neutralizing antibody or PBS immediately after HI insult, twice a day until sacrifice after 48 hours. The ratio of the intact area of 60 μg/kg of anti—LOX-1 neutralizing antibody-treated (60T) brains (Figure 3B) was higher than that of PBS-treated (PBS-T) brains (Figure 3A). The ratios of PBS-T brains and 6 μg/kg of anti—LOX-1 neutralizing antibody-treated (6T) brains were not significantly different from that of HIE brains at 48 hours (Figure 3C). The ratio of 60T brains was significantly higher than those of HIE and PBS-T brains (Figure 3C) and was comparable with that of HT brains. Next, we administered 60 μg/kg of anti—LOX-1 neutralizing antibody twice a day until sacrifice at 24 or 72 hours and assessed the ratios of intact area sequentially. The ratio at 24 and 72 hours in the 60T group was 0.51 ± 0.16 and 0.63 ± 0.17, respectively, being significantly higher than those of HIE (0.31 ± 0.12 and 0.31 ± 0.15) and PBS-T (0.28 ± 0.11 and 0.20 ± 0.07) brains (Figure 3D).

We observed a reduction of HI-induced apoptosis by anti—LOX-1 neutralizing antibody. The number of TUNEL^{+} cells was large in HIE and PBS-T brains (Figures 2A and 4A) but was significantly smaller in 60T brains (Figure 4A). The apoptosis ratio at 48 hours was also reduced in 60T brains

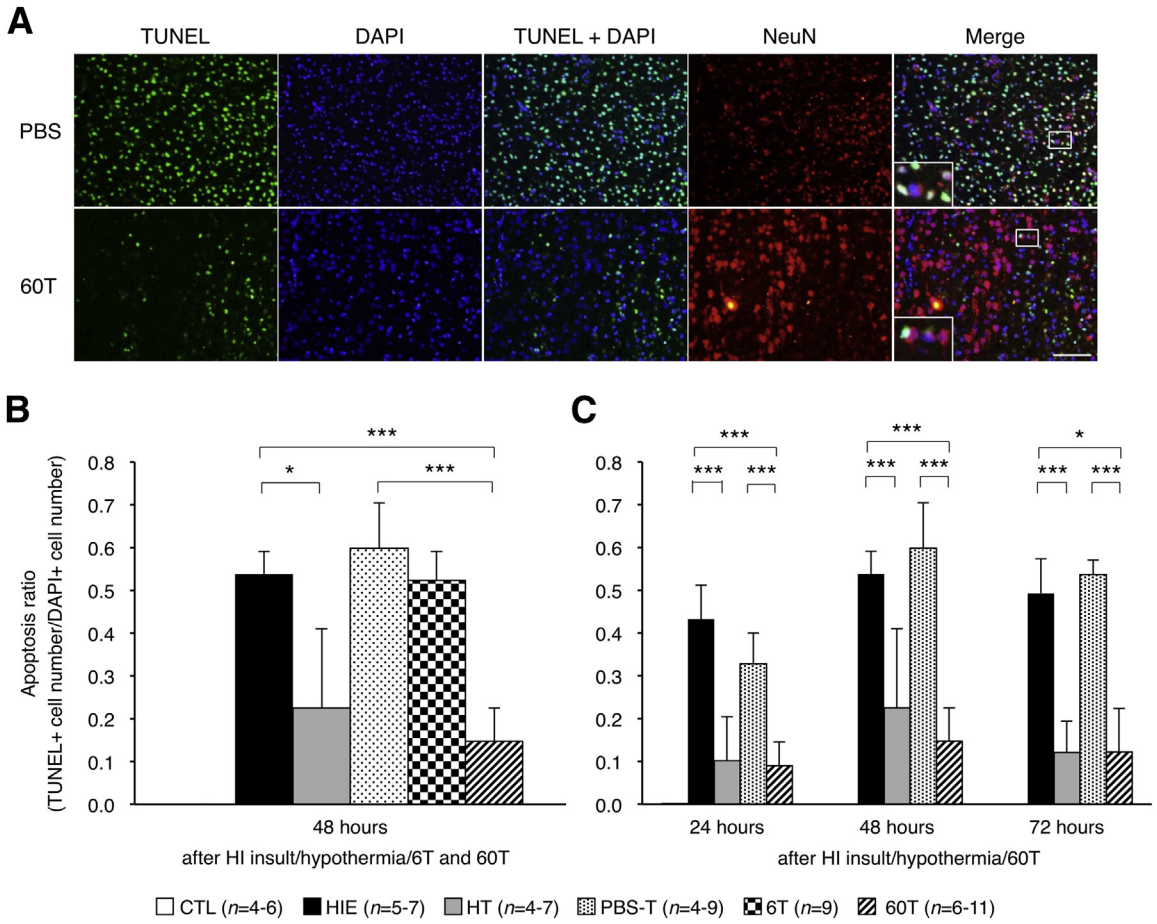

Figure 4 Decreased apoptosis by 60T treatment. **A:** The 60T brain clearly showed a decrease in the number of TUNEL^{+} cells (mainly neurons), compared with the PBS-T brain (48 hours after HI insult). **Insets** are high-power views of rectangular areas. **B:** The apoptosis ratio of 60T significantly decreases, compared with HIE brains, but that of 6T is not different from those of HIE and PBS-T brains (48 hours after HI insult). **C:** The efficacy of 60 μg/kg anti—LOX-1 treatment is noted from 24 hours after HI insult and is comparable with that of HT. Data are expressed as means ± SD. $^{*}P < 0.05$, $^{***}P < 0.001$. Scale bar = 100 μm.

(0.15 ± 0.08), compared with 6T (0.52 ± 0.07), HIE (0.54 ± 0.05), and PBS-T (0.60 ± 0.11) brains ($P < 0.001$) (Figure 4B). The apoptosis ratios of 60T brains at 24 and 72 hours were significantly reduced (0.09 ± 0.06 and 0.12 ± 0.10, respectively), compared with those of HIE (0.43 ± 0.08 and 0.49 ± 0.08) and PBS-T (0.33 ± 0.07 and 0.54 ± 0.03) brains ($P < 0.001$) (Figure 4C).

To examine the antiapoptotic effect of anti−LOX-1 antibody against neonatal HIE, we measured the levels of cCASP3 and MDA. The cCASP3 levels were evaluated due to the relative expression levels by the immunoblotting, compared with those of controls. Thus, we could obtain amount of the unit. No significant differences were found in cCASP3 levels between any groups 24 hours after HI insult (Figure 5A). However, the cCASP3 levels at 48 and 72 hours were significantly lower in 60T brains (0.88 ± 0.33 and 1.29 ± 0.24, respectively) than in HIE brains (1.99 ± 0.94 and 1.69 ± 0.28) ($P < 0.05$). Similarly, the cCASP3 levels at 48 and 72 hours were significantly lower in HT brains (0.96 ± 0.39 and 0.96 ± 0.20) than in HIE brains ($P < 0.05$). MDA levels of 60T brains were 61.70 ± 13.5 pmol/protein (mg) at 24 hours, 91.63 ± 59.6 pmol/protein (mg) at 48 hours, and 84.3 ± 49.0 pmol/protein (mg) at 72 hours (Figure 5B). MDA levels of HIE brains were 97.97 ± 7.09 pmol/protein (mg) at 24 hours, 169.8 ± 59.7 pmol/protein (mg) at 48 hours, and 220.1 ± 92.3 pmol/protein (mg) at 72 hours. MDA levels of the 60T group were significantly lower than those of the HIE group ($P < 0.001$ at 24 hours, $P < 0.05$ at 48 and 72 hours) (Figure 5B).

The edema ratio of 60T brains was significantly lower than that of HIE brains at 48 and 72 hours ($P < 0.05$) (Figure 6A). Moreover, no significant differences were found between HT and 60T brains at any time. The expression of BBB tight junction proteins, occludin and ZO-1, was reduced in HIE brains 48 hours after HI insult ($P < 0.05$) (Figure 6B) but was increased in 60T brains ($P < 0.001$ for occludin, and $P < 0.05$ for ZO-1, compared with HIE brains) (Figure 6C).

Discussion

Every year, a large number of babies experience birth asphyxia, not only in developing countries but also in developed ones. HT has recently been applied to many babies who experienced asphyxia. According to the results of recent clinical trials, it remains difficult for severe cases to survive, but, in moderate cases, brain damage can be alleviated,[7–9,11] showing the neuroprotective effects of HT. The molecular mechanism includes inhibition of excitatory receptor activation, activated caspase to induce apoptotic cell death, mitochondrial failure, free radical damage, lipid peroxidation, and inflammation.[11] Involvement of specific biological pathways has been documented in model animals of neonatal HIE.[11] In this study, we found the role of the LOX-1 pathway in the pathogenesis and treatment of neonatal HIE.

We verified that acute temporary HT in a rat model of neonatal HIE is effective for brain protection. We demonstrated a significant and reproducible reduction in brain damage 24 hours after HI insult with HT. Moreover, we found that HT prevented neuronal apoptosis by reducing oxidative stress and caspase activation and that it ameliorated brain edema by protecting the BBB tight junction. From the HT experiments, we discovered a candidate for a novel therapeutic target, LOX-1 (also known as Olr1). The up-regulation of *Olr1* was closely associated with HI-induced brain damage, and its down-regulation with brain protection by HT. LOX-1 is a receptor of oxidized LDL, binding to various ligands, such as heat shock protein 70 and C-reactive protein.[24–27] In pathological conditions, LOX-1 is up-regulated by the stimuli of tumor necrosis factor-α, oxidant species, and reperfusion after ischemia.[24–27] The activation of LOX-1 induces apoptosis.[24–29] LOX-1 is

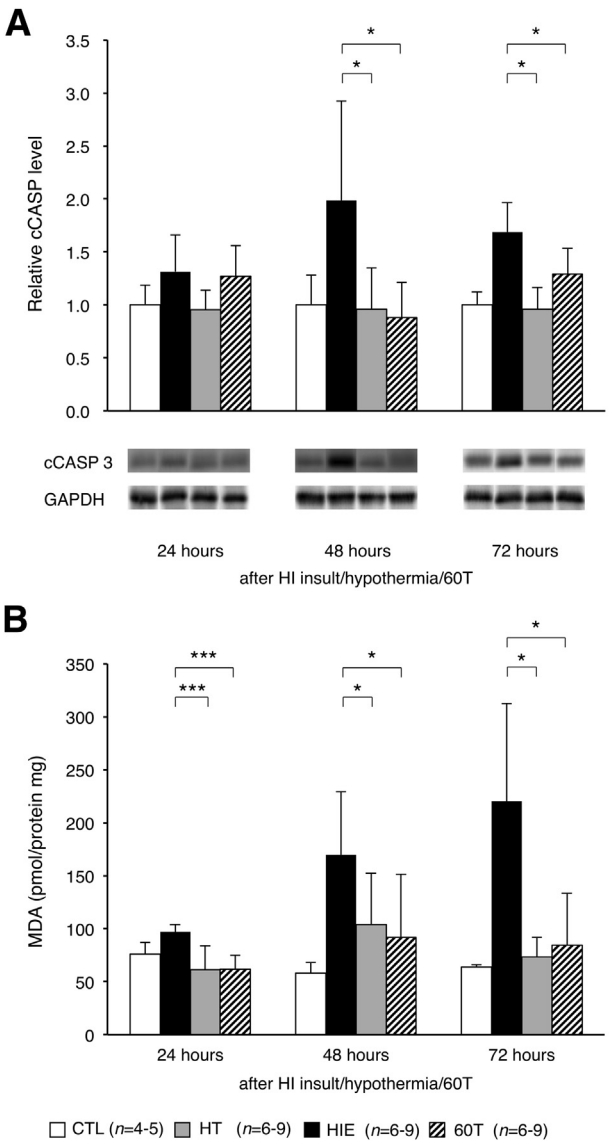

Figure 5 Induction of cCASP3 and MDA expression by anti−LOX-1 neutralizing antibody treatment. **A:** cCASP3 expression is significantly decreased in HT and 60T brains, 48 and 72 hours after HI insult. **B:** MDA is similarly decreased in HT and 60T brains at any time point. Data are expressed as means ± SD. *$P < 0.05$, ***$P < 0.001$.

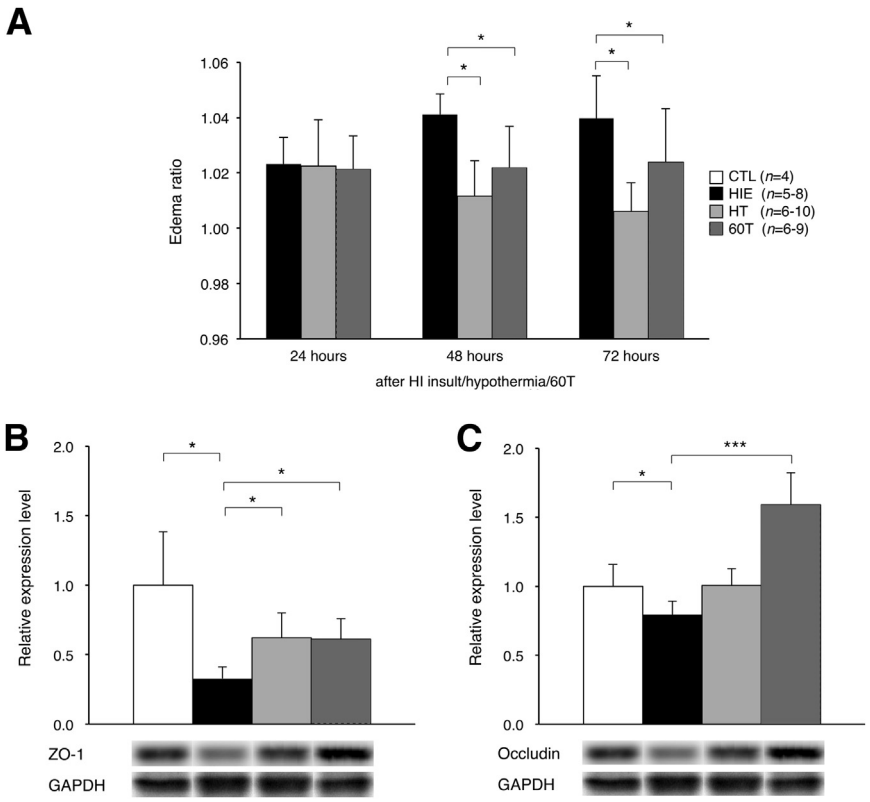

Figure 6 Improvement of brain edema and the recovery of tight junction protein expression by anti—LOX-1 antibody. **A:** Edema ratios are significantly lower in 60T brains, 48 and 72 hours after HI insult. Edema ratios of control brains are 1.00. **B** and **C:** Occludin and ZO-1 levels in the lesional side hemisphere are measured in CTL, HIE, HT, and 60T brains 48 hours after HI insult. Each expression level is standardized by that of GAPDH, and the expression ratio is compared with the CTL expression ratio. Data are expressed as means ± SD. $*P < 0.05$, $***P < 0.001$.

expressed in endothelial cells and vascular smooth muscle cells. Its pathogenetic role in atherosclerosis has been established.[24,25,27,30] As we showed here by *in situ* hybridization, LOX-1 is expressed in neurons. LOX-1 induces neuronal apoptosis in hypertensive rats.[31] An anti—LOX-1 neutralizing antibody reduces LOX-1 concentration and activation. In an atherosclerosis model mouse, this antibody has been successfully applied to improve vascular function in adult animals.[29,32] In our model, the same antibody was also effective for neonatal HIE, and its efficacy was comparable with that of HT. The antibody inhibited both brain edema and neuronal apoptosis. Our data show that one of the mechanisms of its efficacy is BBB protection. Damage of vascular endothelial cells leads to brain edema through incomplete BBB of the neonates' brain. After HI insult, LOX-1 up-regulation induces matrix metalloproteinase expression, which in turn degrades tight junction proteins.[26,33—35] Occludin and ZO-1 are tight junction proteins and major components of BBB, clinically used as markers of BBB breakdown.[36] In this study, ZO-1 expression was up-regulated by both HT and anti—LOX-1 neutralizing antibody. By contrast, occludin expression was up-regulated by anti—LOX-1 neutralizing antibody but not by HT. The LOX-1 signaling pathway may specially regulate occludin expression to maintain BBB. This protection of BBB and the following suppression of brain edema may lead to the inhibition of neuronal apoptosis.

Some neuronal damage in neonatal HIE is caused by the induction of apoptotic cell death through oxidative stress and active caspase. LOX-1 activation increases the amount of cleaved caspase 3, an active form of caspase 3 and a strong apoptosis-inducing protein.[37] MDA is a product of lipid peroxidation from oxidative stress. It directly damages DNA and induces apoptotic cell death. The damage of vascular endothelial cells leads to BBB breakdown, and the neuronal damage leads directly to brain damage. Our results suggest that the antibody-induced reduction of LOX-1 concentration and activation may inhibit damage of vascular endothelial cells and neurons at the early stage of neonatal HIE.

We noted that HI insult and HT altered the expression of 12 genes, encoding cytokines, chemokines, noncoding RNA, transcription factors, and receptors. Among them, we found significant changes in the expression of *P2ry13* and *Olr1* by qPCR and *in situ* hybridization. *P2ry13* encodes P2y13, a receptor of ADP that is expressed in the neuron. It has been reported that the selective agonist of P2y13, 2-methylthioladenosine 5′ diphosphate (2MeSADP), suppresses oxidative stress-induced neuronal death and that 2MeSADP recovered cytotoxic edema and brain infarcts.[22,23] We attempted administration of 2MeSADP in our rats with neonatal HIE, by injecting 0.25, 2.5, or 25 mg/kg 2MeSADP once a day from just after HI insult. However, pathological studies showed no evidence of its efficacy (data not shown). Previous studies have shown that active P2y13 inhibits neuronal differentiation and even induces apoptosis.[38,39] Taken together, it may be difficult to use P2y13 as a therapeutic target.

Our neonatal HIE rat has been modified and widely used as a standard of neonatal HIE model.[2,18] The rat brain at

postnatal day 7 is known to be histologically similar to that of a term human fetus or newborn infant, showing complete cerebral cortical neuronal layering, involute germinal matrix, and little myelination of white matter.[40] Thus, the rat brain at postnatal day 7 is reasonable as the HIE model. Of course, one must challenge other models to provide firm treatment with anti−LOX-1 antibody.

Conclusion

Our study identified LOX-1 as a novel therapeutic target for neonatal HIE. Although many problems need to be addressed before its clinical application, the inhibition of LOX-1 may become a novel treatment for babies who experienced asphyxia in the near future.

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Supplemental Data

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